

Catalase, Peroxidase, and Polyphenoloxidase Activities during Rice Leaf Senescence¹

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ABSTRACT

The activities of catalase, peroxidase, and polyphenoloxidase were studied in attached and detached rice (*Oryza sativa* L. cv. Ratna) leaves. Catalase activity decreased while peroxidase and polyphenoloxidase activities increased during senescence of both attached and detached rice leaves. Kinetin (5 μ M) and benzimidazole (1 mM), which are known to delay the senescence of detached rice leaves, retarded the decrease of catalase activity during detached leaf senescence. On the other hand, these chemicals accelerated the increase of peroxidase and polyphenoloxidase activities over the water control. Total phenolics accumulated in detached and darkened rice leaves, but in attached leaf senescence in light no accumulation of phenolics was observed.

Detached leaves or leaf disks floated on water are characterized by drifts in the activities of various enzymes (3, 4, 6, 7, 10, 13, 15) and an increase in the respiratory rate (21, 23). Manifold increase in the activities of several oxidative enzymes during detached leaf senescence has been reported (4, 7, 13). The majority of the observations support the idea that senescence and higher levels of oxidative enzymes are closely associated phenomena, but contradictory reports have also appeared. For example, a group of Hungarian workers (4, 7) reported that the catalase (EC 1.11.1.6) activity in tobacco leaves decreased but in wheat and barley leaves it increased upon detachment. Parish (13) reported a decrease in the activity of catalase in tobacco leaves during senescence and maturation. There are several reports that peroxidase (EC 1.11.1.7) activity increases during senescence of detached leaves or leaf disks (4, 7, 13). Increase in the activity of this enzyme with the physiological age of the leaves has also been reported (5, 9, 13). Parish (13) also suggested that the increase in the activity of peroxidase is one of the most reliable indicators of maturity and senescence. But Ford and Simon (5) contradicted Parish's (13) suggestion because peroxidase activity increased several-fold when senescence was delayed and Chl and protein levels increased in the cotyledons of detopped cucumber seedlings. They concluded from these observations that at least in this particular case the rise in peroxidase activity cannot be taken as a reliable indicator of senescence. Polyphenoloxidase (EC 1.10.3.1) activity in-

creases during senescence of detached leaves or leaf disks (4, 7, 14) as well as with the physiological age of the attached leaves (9).

In view of these conflicting reports concerning catalase, peroxidase, and polyphenoloxidase activities during senescence and aging of leaves, the present investigation was designed to assay these enzymes during induced senescence of excised rice leaves as well as during the normal senescence of the attached rice leaves.

MATERIALS AND METHODS

Seeds of a high yielding variety of rice (*Oryza sativa* L. cv. Ratna) were obtained from the Central Rice Research Institute, Cuttack, and the plants were grown in field conditions. Leaves from 8-week-old rice plants were used.

Seven-centimeter tips from fully expanded and matured leaves were washed in distilled H₂O, randomized, and floated in groups of five (weighing about 200 mg) in 30 ml of distilled H₂O (control), (5 μ M) kinetin or (1 mM) benzimidazole solution in 10-cm Petri dishes in the dark at room temperature (25 \pm 3C). Samples were taken initially and at intervals for biochemical and enzymic analyses.

Enzyme Extraction and Assay. The leaf samples, weighing about 200 mg, were homogenized with 10 ml of phosphate buffer pH 6.8 (0.1 M) and divided into two equal 5-ml portions. One 5-ml portion was centrifuged at 2 C for 15 min at 17,000g in a refrigerated centrifuge. The clear supernatant was taken as the enzyme source. The other 5-ml portion was taken for the biochemical analysis.

Catalase Assay. The activity of catalase as well as peroxidase was assayed after the method of Chance and Maehly (2) with the following modifications.

Five milliliters of the assay mixture for the catalase activity comprised: 300 μ moles of phosphate buffer, pH 6.8, 100 μ moles of H₂O₂, and 1 ml of the twice diluted enzyme extract. After incubation at 25 C for 1 min, the reaction was stopped by adding 10 ml of 2% (v/v) H₂SO₄ and the residual H₂O₂ was titrated against 0.01 N KMnO₄ until a faint purple color persisted for at least 15 sec. A control was run at the same time in which the enzyme activity was stopped at "zero" time. One unit of catalase activity is defined as that amount of enzyme which breaks down 1 μ mol of H₂O₂/min under the assay conditions described.

Peroxidase Assay. Five milliliters of the assay mixture for the peroxidase activity comprised: 125 μ moles of phosphate buffer, pH 6.8, 50 μ moles of pyrogallol, 50 μ moles of H₂O₂, and 1 ml of the 20 times-diluted enzyme extract. This was incubated for 5 min at 25 C after which the reaction was stopped by adding 0.5 ml of 5% (v/v) H₂SO₄. The amount of purpurogallin formed was determined by taking the absorbancy at 420 nm.

Polyphenoloxidase Assay. Five-milliliter assay mixture for

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polyphenoloxidase activity consisted of the same assay mixture as that of peroxidase without H_2O_2 . The absorbancy of the purpurogallin formed was taken at 420 nm.

Peroxidase and polyphenoloxidase activities were expressed in absorbancy units.

Biochemical Analysis. The 5-ml portion of the homogenate was mixed with four parts of ethyl alcohol so that the final concentration of ethyl alcohol became 80% (v/v). This was boiled in a water bath for 10 min, cooled, and centrifuged. The pellet was extracted three times with boiled ethyl alcohol and centrifuged. The supernatants were combined and made to volume. Chlorophyll was determined spectrophotometrically at 665 nm. α -Amino nitrogen was determined from the alcoholic extract by the Moore and Stein method (12). Total phenolics was determined from the alcoholic extract by the phenol reagent method (18).

The pellet was washed successively with 10% (w/v) cold trichloroacetic acid (twice), ethyl alcohol (once), ethyl alcohol-chloroform (3:1, v/v, twice), ethyl alcohol-ether (3:1, v/v, once), and finally with ether (once). The pellet was evaporated to dryness. The protein was solubilized by boiling with 1 N NaOH for 15 min in a water bath. It was centrifuged and an aliquot was taken for protein determination (8).

Chlorophyll, α -amino nitrogen, protein, and total phenolics were expressed in absorbancy units.

RESULTS

Biochemical Parameters of Senescence. The biochemical parameters generally used to determine the senescence process in detached leaves are the decrease in the Chl and protein content and an increase in the α -amino nitrogen content (10). When the excised rice leaves were floated on water in the dark, these gradually turned yellow because of the loss of Chl content with time (Fig. 1). This loss in the Chl content was accompanied by the loss in the protein content (Fig. 2) with the concomitant rise in the α -amino nitrogen level (Fig. 3). Kinetin and benzimidazole are known to delay the senescence of detached leaves (6, 11) and when the excised leaves were floated on kinetin or benzimidazole solution, the general decline in the Chl (Fig. 1) and protein (Fig. 2) levels was arrested. The increase in the α -amino nitrogen level was also prevented by these treatments (Fig. 3). There was a positive correlation at 99.9% level ($r = 0.9429$, $n = 10$) between the Chl content and the protein content during the senescence of detached leaves. An extremely high negative correlation at 99.9% level was obtained between Chl and α -amino nitrogen levels ($r = -0.9280$, $n = 10$) and between

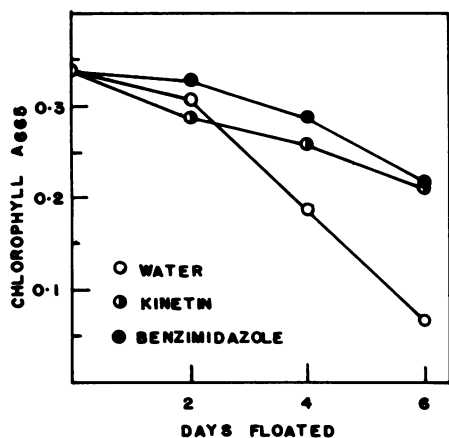


FIG. 1. Changes in the Chl content of rice leaves with time after detachment. The detached leaves were floated on water, $5 \mu\text{M}$ kinetin, or 1 mM benzimidazole solutions in dark. (The leaf treatment was the same for Figs 2, 3, 4, 6, 8, 10).

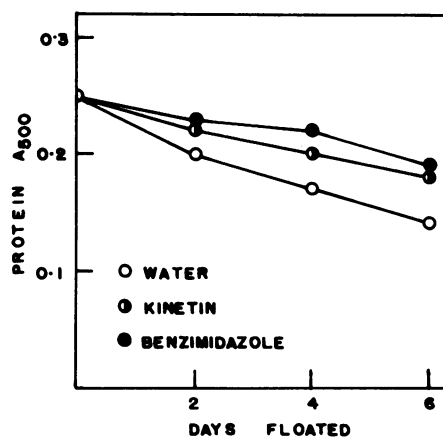


FIG. 2. Changes in the protein content of rice leaves with time after detachment.

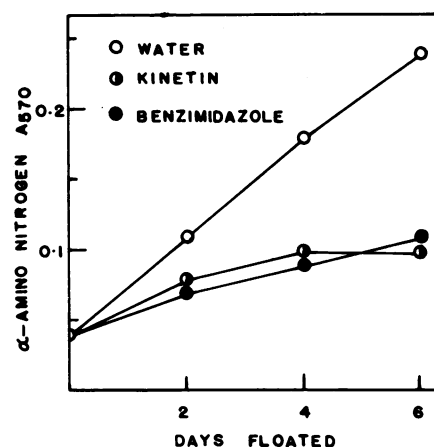


FIG. 3. Changes in the α -amino nitrogen content of rice leaves with time after detachment.

protein and α -amino nitrogen levels ($r = -0.9305$, $n = 10$).

Senescence of an organ attached to the whole plant is not essentially the same as the senescence of the organ detached from the plant. Some differences are to be expected, and in fact some modest differences have been reported between the senescence of attached and detached apple leaves (16) and oat leaves (20).

An attempt has been made to assess how the three parameters used to determine the senescence process hold good for the attached aging rice leaves. Table I shows the levels of Chl, protein, and α -amino nitrogen in attached aging rice leaves. The variety of rice under study was a dwarf variety and it had only four leaves in the main tiller during the panicle-bearing stage, *i.e.*, at the time when the flag leaf was fully matured. The leaves were numbered from the apex to the base. The leaf insertion level is the function of the physiological age of the leaf and the age of the leaf increased as it is sampled from the apex downward. Both the Chl and protein contents decreased with the increased age of the leaves. In the fourth leaf the Chl and protein contents were about 50% of the flag leaf (first leaf). It can be recalled that during detached leaf senescence, both Chl and protein contents also decrease with time. But in contradiction to the detached leaves, α -amino nitrogen failed to accumulate in the attached aging leaves and actually the fourth leaf had about 50% of the α -amino nitrogen content of the first leaf. This low level of α -amino nitrogen in senescent attached leaves is primarily due to the continuous transport of the amino acids to the surrounding active tissues as previously reported (20). The higher level of α -amino nitrogen in the flag leaf is probably due

to the synthesis of amino acids from the photosynthetic intermediates.

ENZYMATIC CHANGES DURING DETACHED LEAF SENESCENCE

Catalase. When the leaves were excised and floated on water in dark the catalase activity gradually declined with time (Fig. 4). At the 6th day the total catalase activity was about 55% of the initial activity. Kinetin and benzimidazole at the concentrations known to retard senescence retarded the decrease in the catalase activity. At the 6th day the kinetin- and benzimidazole-treated leaves had about 83 and 86.5% of the initial activity, respectively. The decrease of the catalase activity is positively correlated at the 99.9% level ($r = 0.9571$, $n = 10$) with the decrease of the Chl content during senescence (Fig. 5).

Peroxidase. Figure 6 shows the changes in the peroxidase activity during detached leaf senescence and the effect of benzimidazole and kinetin. Peroxidase activity increased up to 2 days after which the increase was quite slow. Kinetin and benzimidazole at the concentrations known to delay the senescence of detached rice leaves further enhanced the peroxidase activity. This enhancement of peroxidase activity over the water control was more prominent in the kinetin-treated leaves than in the benzimidazole-treated leaves. Chlorophyll content did not give a good correlation with the peroxidase activity ($r = -0.1911$, $n = 10$) during senescence of detached leaves (Fig. 7).

Polyphenoloxidase. During detached-leaf senescence polyphenoloxidase activity increased with time in the water-floated leaves (Fig. 8). At the 6th day the increase was 50% over the initial activity. When detached leaves were floated on kinetin or benzimidazole solution the enzyme activity was further increased and the treated leaves had enzyme activity higher than the water-floated leaves. This enhancement of polyphenoloxidase activity by kinetin and benzimidazole was not so prominent as in the case of peroxidase activity. Regression analysis showed that polyphenoloxidase activity was negatively corre-

Table I. Chlorophyll, Protein, and α -Amino Nitrogen Content of Attached Senescing Rice Leaves as Function of Leaf Insertion Level
The leaves were numbered from the apex to the base.

Leaf Position	Chl	Protein ¹	α -Amino Nitrogen
	A_{665}	A_{500}	A_{570}
1, Flag leaf	0.36	0.27	0.16
2	0.32	0.22	0.13
3	0.24	0.16	0.08
4	0.17	0.14	0.08

¹ To obtain protein value in mg per g fresh weight of leaves each absorbancy value should be multiplied by 250.

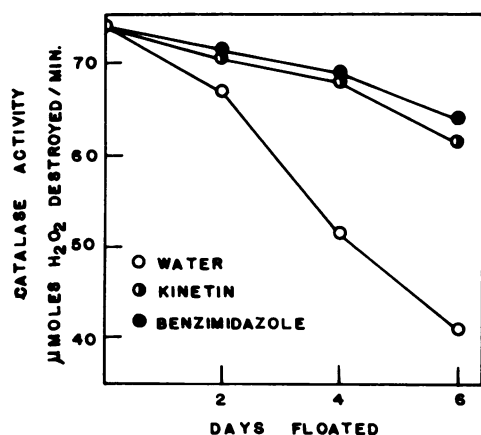


FIG. 4. Changes in the catalase activity of rice leaves with time after detachment.

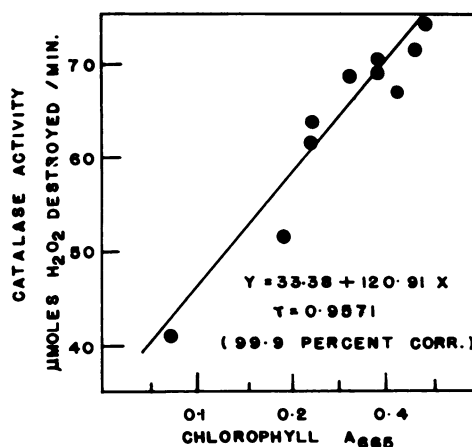


FIG. 5. Correlation between catalase activity and Chl content of detached senescing rice leaves.

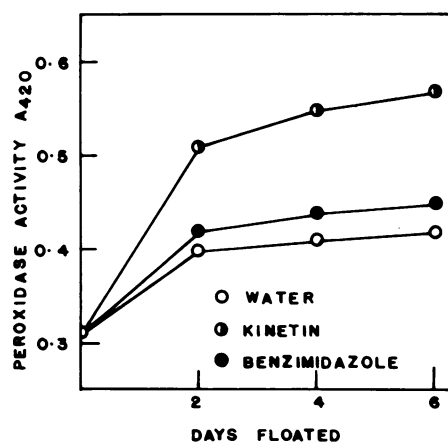


FIG. 6. Changes in the peroxidase activity of rice leaves with time after detachment.

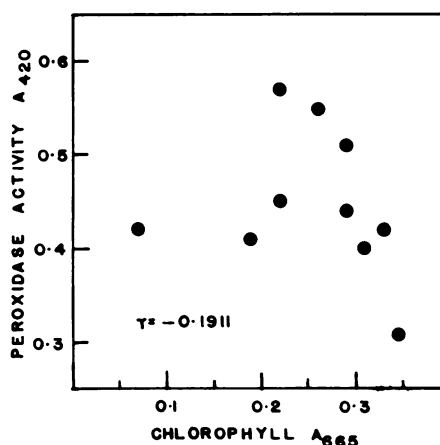


FIG. 7. Correlation between peroxidase activity and Chl content of detached senescing rice leaves.

lated (Fig. 9) with the Chl content at the 95% level ($r = -0.6552$, $n = 10$).

Enzymatic Changes during Senescence of Attached Leaves. Enzyme assays were made in attached rice leaves of different ages and the enzyme activities as the function of the leaf insertion level are presented in Table II. It may be seen that the senescence of attached rice leaves was associated with a steady decrease in the catalase activity. On the other hand, peroxidase and polyphenoloxidase activities increased as the leaves were

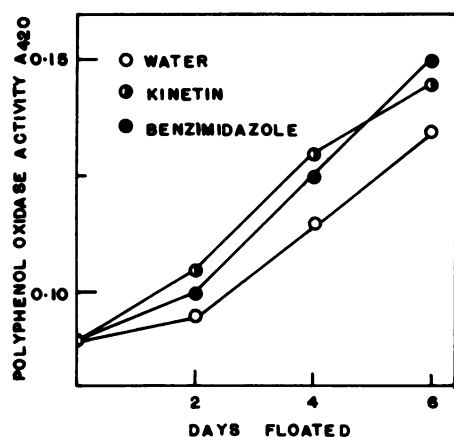


FIG. 8. Changes in the polyphenoloxidase activity of rice leaves with time after detachment.

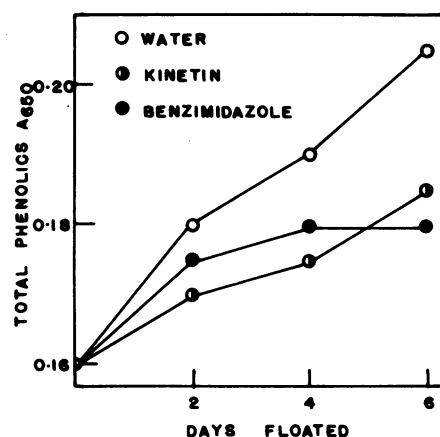


FIG. 10. Changes in the total phenolics content of rice leaves with time after detachment.

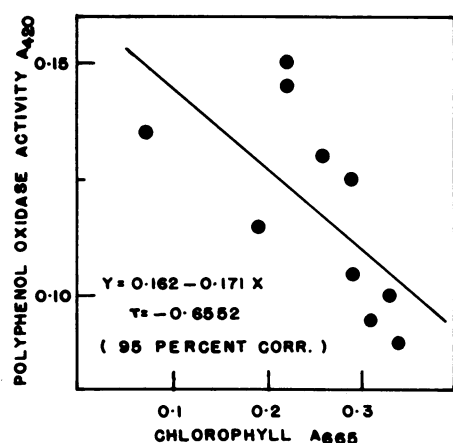


FIG. 9. Correlation between polyphenoloxidase activity and chlorophyll content of detached senescing rice leaves.

Table II. Catalase, Peroxidase, and Polyphenoloxidase Activities in Attached Senescing Rice Leaves as Function of Leaf Insertion Level. The leaves were numbered from the apex to the base.

Leaf Position	Catalase $\mu\text{moles H}_2\text{O}_2$ destroyed/min	Peroxidase A_{420}	Polyphenoloxidase A_{420}
1, Flag leaf	77.5	0.300	0.095
2	68	0.355	0.105
3	55.5	0.405	0.120
4	38	0.420	0.125

sampled from the apex downward. The fourth leaf has about 50% of catalase activity than that of the first leaf. In the fourth leaf peroxidase and polyphenoloxidase activities increased to 40 and 31%, respectively, over the first leaf.

Changes in the Total Phenolics during Senescence. As the phenolics are the substrates for the peroxidase and polyphenoloxidase enzymes, an attempt was made to determine the level of total phenolics during the senescence of detached and attached leaves. Upon detachment the phenolic content increased with time (Fig. 10). At the 6th day there was only a 28% increase of the total phenolics over the initial value. Both kinetin and benzimidazole treatments decreased the increasing phenolic content during detached leaf senescence. There was negative correlation at 99.9% level ($r = -0.9012$, $n = 10$) between Chl and total phenolics levels. Total phenolics showed no change in concentrations with leaves of different ages. The four leaves

from the apex to the base contained 0.18, 0.19, 0.17, and 0.18 absorbancy units of total phenolics, respectively.

DISCUSSION

In the present investigation we noticed that catalase activity decreased while peroxidase and polyphenoloxidase activities increased during senescence of detached leaves. In attached leaves, also, catalase activity decreased with the progress of senescence while peroxidase and polyphenoloxidase activities increased. Kinetin and benzimidazole treatments delayed the decrease of the catalase activity but enhanced the peroxidase and polyphenoloxidase activities over the water control. Both the catalase and peroxidase enzymes have iron-protoporphyrin as their prosthetic group. In spite of this common prosthetic group, it is not clear why the activities of these enzymes during senescence showed opposite trends to each other.

A number of controversial results have been published concerning the catalase activity during detached leaf senescence. Both increased and decreased levels of catalase activity have been reported. For example, catalase activity decreased in tobacco (4, 13) and increased in wheat and barley (4, 7) during detached leaf senescence. In the present study this enzyme decreased during senescence of both attached and detached leaves of rice. Apparently the trend of changes in catalase activity during senescence is species specific; its activity increases during senescence in some plants and decreases in others.

The level of total phenolics increased in the detached leaves floated on water and kinetin and benzimidazole treatments inhibited the increase of the total phenolics during senescence. Increase of the total phenolics upon detachment and their inhibition by kinetin treatment have also been reported previously (23). The increase of the total phenolics during senescence may account for the release of the phenolics from the leaky vacuoles and the liberation of the phenolic amino acids from proteolysis.

Polyphenoloxidase activity is absent in rice leaves (18, 22) despite repeated efforts to show its presence using substrates other than pyrogallol. But Varga (24) detected a polyphenoloxidase activity in rice leaves employing pyrogallol as the substrate. Our study shows that polyphenoloxidase in rice leaves utilizes pyrogallol as substrate and it has pH optima between pH 6.5 and 7. That the oxidation of pyrogallol is due to a potential enzyme activity was established by the fact that the enzyme extract boiled for 5 min failed to oxidize pyrogallol, whereas the fresh enzyme did so quite rapidly.

As peroxidase and polyphenoloxidase enzymes catalyze the oxidation of the same types of compounds, in general, plants

contain either peroxidase or polyphenoloxidase, activity but rarely both (1). Therefore, as the rice leaves contain a potential peroxidase activity, the functional significance of polyphenoloxidase activity in these leaves is not clear. It has been suggested that different isoenzymes of polyphenoloxidase show different substrate specificity (22). During the course of evolution perhaps all but the pyrogallol-specific isoenzymes of polyphenoloxidase have been degenerated in rice plants.

Whether the increase in peroxidase activity during senescence should be taken as a reliable indicator of senescence is a debatable point (5, 13). If the rise in any enzyme activity is of functional significance during senescence process, then kinetin and benzimidazole should prevent the increase, *i.e.*, the effects of these agents on the enzyme activity should be opposite to their effects on Chl and protein contents of detached leaves (6, 10). But contrary to this theoretical view, the increase in the peroxidase activity during detached leaf senescence is further enhanced by kinetin and benzimidazole treatment. The indication is that perhaps peroxidase has no functional significance during the senescence process. Its increase during senescence is due to the effect of wounding caused upon detachment of the leaves. Therefore, the increase in peroxidase activity should not be taken as a reliable indicator of senescence.

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